Activation of c-Jun N-Terminal Kinase Cascades Is Involved in Part of the Neuronal Degeneration Induced by Trimethyltin in Cortical Neurons of Mice

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Received August 12, 2008; Accepted November 6, 2008

Abstract. The organotin trimethyltin (TMT) is known to cause neuronal degeneration in the central nervous system. A systemic injection of TMT produced neuronal damage in the cerebral frontal cortex of mice. To elucidate the mechanism(s) underlying the toxicity of TMT toward neurons, we prepared primary cultures of neurons from the cerebral cortex of mouse embryos for use in this study. Microscopic observations revealed that a continuous exposure to TMT produced neuronal damage with nuclear condensation in an incubation time–dependent manner up to 48 h. The neuronal damage induced by TMT was not blocked by N-methyl-D-aspartate receptor channel–blocker MK-801. The exposure to TMT produced an elevation of the phosphorylation level of c-Jun N-terminal kinase (JNK)p46, but not JNKp54, prior to neuronal death. Under the same conditions, a significant elevation was seen in the phosphorylation level of stress-activated protein kinase 1, which activates JNKs. Furthermore, TMT enhanced the expression and phosphorylation of c-Jun during a continuous exposure. The JNK inhibitor SP600125 was effective in significantly but only partially attenuating the TMT-induced nuclear condensation and accumulation of lactate dehydrogenase in the culture medium. Taken together, our data suggest that the neuronal damage induced by TMT was independent of excitotoxicity but that at least some of it was dependent on the JNK cascades in primary cultures of cortical neurons.

Keywords: c-Jun N-terminal kinase, cortical neuron, neuronal death, stress-activated protein kinase 1, trimethyltin

Introduction

The molecular basis of selective vulnerability of specific neuronal populations to neuronal insults has been a key focus in neurology and neuropathology. The organotin trimethyltin chloride (TMT), which had been used as a fungicide and chemostabilizer, selectively induces neuronal damage in both human and rodent central nervous systems (1). The pattern of neurodegeneration elicited by a single treatment with TMT is different between rats and mice: in rats, TMT-induced damage is first seen in the granule cells of the dentate gyrus and then in the pyramidal cells of the CA1 and CA3c/CA4 subfields (2, 3), whereas in adult mice, the damage occurs exclusively in the granule neurons of the dentate gyrus (4–8). In addition to the dentate gyrus, the olfactory bulb and anterior olfactory nucleus of the mice show neuronal injury induced by TMT (9). However, the exact reason for the vulnerability of the neurons in specific brain regions to TMT toxicity remains unclear.

Transcription of genes in neurons and glia is activated as a response to the damage due to exposure to various neurotoxins, stroke, trauma, or ischemia. Under these
injurious conditions, the fate of neurons, that is, death or survival, is determined by newly expressed genes related to apoptosis, regeneration, and repair. The transcription factor activator protein-1 (AP-1) has been implicated in these processes (10, 11). AP-1 is a homodimeric and/or heterodimeric protein complex consisting of c-Fos and c-Jun family proteins (12). Although c-Jun is involved in multiple cellular functions, such as survival, differentiation, and regeneration, other evidence points to its involvement in neuronal apoptosis (13). The activity of AP-1 is regulated by post-translational phosphorylation and/or dephosphorylation of c-Jun, the former effected by c-Jun N-terminal kinase (JNK) acting at the NH$_2$-terminal site of c-Jun (14, 15). The JNK pathway is activated in response to free radicals generated by UV radiation, exposure to inflammatory cytokines, and direct application of H$_2$O$_2$ (15), as well as in response to dopamine (16) and glutamate (17). Further evidence for the involvement of JNK in neuronal death comes from many reports: overexpression of dominant-negative components of the JNK pathway reverses apoptosis induced by withdrawal of trophic factors in PC12 cell cultures (18), knockout of the JNK3 gene reduces kainate-induced pyramidal cell death in the mouse hippocampus (19), and an inhibitor of JNK prevents activation of caspases and apoptosis of cultured cerebellar granule neurons (20). Upstream activators of JNK have been identified by investigations conducted over the past few decades. JNKs are activated through phosphorylation of their threonine 183 and tyrosine 185 by stress-activated protein kinase 1 (SEK1), which is also known as extracellular-signal regulated kinase kinase or mitogen-activated protein kinase kinase (MKK) 4, and by MKK7 (also known as SEK2; 21, 22). Furthermore, it is known that SEK1 is activated by the phosphorylation of serine 219 and threonine 261 (23).

Recent in vivo experiments of ours demonstrated neuronal death and the activation of JNK signaling in the dentate gyrus following a single systemic injection of TMT (24). However, no convincing evidence has been provided regarding the involvement of JNK cascades in TMT-induced neuronal degeneration. Therefore, the objective of the present study was to investigate whether the JNK pathway is activated as a signal for TMT-induced damage of primary neuronal cultures prepared from the cerebral cortex of mouse embryos. Our present findings indicate that a JNK cascade was activated at an early stage in TMT-induced neuronal death. Blockage of this cascade prevented neuronal death induced by TMT, indicating the involvement of JNK cascades in the toxicity of TMT toward the cortical neurons.

Materials and Methods

Materials

Antibody diluent was purchased from DAKO (Glostrup, Denmark); cytosine-$\beta$-D-arabinofuranoside was from Sigma-Aldrich (St. Louis, MO, USA). Other materials used and their sources were as follows: 0.02% EDTA solution, Dulbecco’s modified eagle medium (DMEM), and 1:1 mixture of DMEM and Nutrient mixture F-12 (DMEM/F12), from Invitrogen (Carlsbad, CA, USA); penicillin-streptomycin solutions, Hoechst 33342, and poly-L-lysine, from Nacalai Tesque Inc. (Kyoto); TMT and Rapid Protein Assay kit, from Wako Pure Chemical Industries, Ltd. (Osaka); polyvinylidene fluoride membranes (Immobilon-P), from Millipore (Bedford, MA, USA); Western Lightning Chemoluminescence Reagent Plus, from PerkinElmer Life Science Products (Boston, MA, USA); X-ray film, from FujiFilm (Tokyo). In addition, the immuno-reagents listed below were also used.

Primary antibodies: monoclonal antibodies against microtubule associated protein (MAP-2; Chemicon International, Temecula, CA, USA), glial fibrillary acidic protein (GFAP; Sigma Chemicals, St. Louis, MO, USA), and $\beta$-tubulin III (CYMBUG Biotechnology, Southampton, UK); polyclonal antibodies against c-Jun (Santa Cruz Biotechnology, Santa Cruz, CA, USA); polyclonal antibodies against SEK1 (#9152), p-SEK1 (#9151), SAPK/JNK (#9252), phospho-SAPK/JNK (#9251), phospho-c-Jun (#9164) (Cell Signaling Technology Inc., Danvers, MA, USA); and single stranded DNA (ssDNA, Dako Denmark A/S, Glostrup, Denmark). Secondary antibodies: anti-mouse IgG antibody conjugated with fluorescein isothiocyanate (Sigma Chemicals, St. Louis, MO) for immunocytochemical analysis, goat anti-mouse IgG antibody conjugated with horseradish peroxidase (GE Healthcare UK Ltd., Buckinghamshire, UK) and anti-mouse or rabbit IgG antibody conjugated with horseradish peroxidase (DAKO Denmark A/S, Glostrup, Denmark) for immunoblot analysis. All other chemicals used were of the highest purity commercially available.

Drug administration in vivo

The protocol used here met the guidelines of the Japanese Society for Pharmacology and was approved by the Committee for Ethical Use of Experimental Animals at Setsunan University. All efforts were invariably made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques. Adult male Std-ddY mice, weighing 30 – 35 g and 5 – 6 weeks of age, were purchased from a local supplier and housed in metallic breeding cages in a
room with a light-dark cycle of 12 h/12 h, a humidity of 55%, and a temperature of 23°C, with free access to food and water for at least 4 days before use. The animals were intraperitoneally injected with TMT (2.8 mg/kg) dissolved in phosphate-buffered saline and then returned to their home cages until the time of decapitation.

**Histological assessment of the cerebral cortex slices**

Mice were deeply anesthetized with pentobarbital (250 mg/kg, i.p.) and perfused via the heart with saline, followed by 4% (wt/vol) paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). The brains were quickly removed and further fixed with the same fixative solution at 4°C overnight. Post-fixed brains were embedded in paraffin, cut as coronal sections of 2-μm thickness with a microtome, and placed on Matsunami adhesive silane–coated slide glasses (Matsunami Glass Ind., Ltd., Kyoto). The paraffin-embedded brain sections were deparaffinized with xylene, rehydrated by immersion in ethanol of graded decreasing concentrations of 100% to 50% (vol/vol), and finally washed with water.

For detection of DNA fragmentation, ssDNA immunoreactivity in coronal sections was determined. Sections were washed with TBST and then incubated with 0.03% (vol/vol) H₂O₂ in methanol for 5 min. After having been blocked with 5% (vol/vol) normal goat serum in TBST for 1 h at room temperature, the sections were incubated with an anti-ssDNA antibody (1:1000) at 4°C overnight. After another wash with TBST, these sections were then reacted with biotinylated anti-rabbit IgG antibody (1:200) for 30 min at room temperature and then reacted for 2 h at room temperature with the appropriate secondary antibody. Finally, the cells were incubated with the desired primary antibody for 2 h at room temperature and subsequently incubated with 1% (wt/vol) methyl green solution (pH 4.0).

**Cell cultures**

Primary cultures of cortical neurons were prepared from 15-day-old embryonic ddY mice as originally described by di Porzio et al. (25) with some modifications. In brief, the dissected cortex was incubated for 12 min at room temperature in 0.02% (wt/vol) H₂O₂ in methanol for 5 min. After having been blocked with 5% (vol/vol) normal goat serum in TBST for 1 h at room temperature, the sections were incubated with an anti-ssDNA antibody (1:1000) at 4°C overnight. After another wash with TBST, these sections were then reacted with biotinylated anti-rabbit IgG antibody (1:200) for 30 min at room temperature and subsequently with the reagents of the streptavidin-biotin complex peroxidase kit for 1 h at room temperature. The peroxidase reaction was visualized with diamino-benzidine/hydrogen peroxide solution. Counter staining was performed by use of 1% (wt/vol) methyl green solution (pH 4.0).

**Histological assessments**

Cultured cells were washed with Tris-buffered saline containing 0.05% (wt/vol) Tween 20 (TBST) and fixed with 4% (wt/vol) paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 20 min at 4°C. After having been subsequently blocked with 5% (vol/vol) normal goat serum in TBST for 1 h at room temperature, they were then incubated with a primary antibody at 4°C overnight. After a wash with TBST, the cells were then reacted for 2 h at room temperature with the appropriate secondary antibody. Finally, the cells were observed under a fluorescence microscope (U-LH100HG; Olympus, Osaka).

Shrunken cells with nuclear condensation were observed as damaged cells by the focal fluorescence of Hoechst 33342 incorporated into the nuclei. After exposures to TMT for various periods to time, the cells were fixed with 4% paraformaldehyde for 20 min and then treated with 10 mg/mL Hoechst 33342 for 20 min at room temperature. The number of damaged cells was counted under the fluorescence microscope. Cells were counted in 5 different visual fields randomly selected on each coverslip. The number of cells was determined as the average of those found in the visual fields.

**Immunoblot analysis**

After treatment with chemicals for various periods, cortical neurons were lysed and boiled for 5 min in the presence of 2% (wt/vol) SDS, 5% (vol/vol) 2-mercaptoethanol, 10% (vol/vol) glycerol, and 0.01% (wt/vol) bromophenol blue immediately after preparation and then stored at −80°C until used for immunoblot analysis, which was carried out as described previously (26). Briefly, an aliquot (20 μg protein) of sample was loaded onto a 10% (wt/vol) polyacrylamide gel, electrophoresed, and transferred to a polyvinylidene fluoride membrane. After having been blocked with 5% (wt/vol) skim milk dissolved in TBST, the membranes were incubated with the desired primary antibody for 2 h at room temperature and subsequently incubated with 37°C in 95% air / 5% CO₂. At 2 days in vitro (DIV), the cells were treated with cytosine-β-D-arabinofuranoside at 5 μM for 24 h to avoid the growth of proliferative contaminants such as glial cells. The neurons were then maintained in the serum-containing medium until 6 DIV and subsequently maintained in the same medium lacking serum but supplemented with 50 μg/mL transferrin, 500 ng/mL insulin, 1 pM β-estradiol, 3 nM triiodothyronine, 20 nM progesterone, 8 ng/mL sodium seleniate, and 100 μM putrescine. At 9 DIV, the cells were treated with 5 μM TMT for various periods of incubation.
horseradish peroxidase–conjugated secondary antibodies for 1 h at room temperature. Proteins reactive with the antibody were detected with the aid of Western Lightning Chemoluminescence Reagent Plus and exposure to X-ray films.

**LDH release**

Accumulation of lactate dehydrogenase (LDH) in culture medium of each dish was measured after treatment with chemicals for 48 h. The medium from each culture dish was collected and stored at −80°C as a sample until assayed for its content of LDH. The samples were mixed with 4 volumes of 0.1 mM NADH in 0.1 M KH₂PO₄ (adjusted to pH 7.5 by KOH), and then immediately 100 mM pyruvate was added to each mixture to start the reaction. At 90 s after the addition of the pyruvate, the absorption of the mixture at 340 nm was measured at 30-s intervals until 300 s from the start. The experiments were performed in triplicate.

**Data analyses**

All data were expressed as the mean ± S.E.M., and the statistical significance was determined by using the two-tailed Student t-test or one-way ANOVA with the Bonferroni/Dunnett post hoc test. Densitometric analysis for quantification in certain assays was carried out with the aid of Atto Densitograph software (Atto Co., Tokyo).

**Results**

**TMT treatment produced neuronal death in the cerebral cortex of mice in vivo**

To assess neuronal damage in the cerebral cortex after a single injection of TMT at the above dose, we performed immunostaining for ssDNA on the frontal cerebral cortex sections prepared from mice injected with TMT. On day 2 post TMT treatment, cells immunoreactive toward anti-ssDNA antibody were found in the cerebral frontal cortex (Fig. 1), in the addition to the dentate gyrus, olfactory bulb, and anterior olfactory nucleus (5–9). Neuronal loss was also observed in the cerebral cortex on day 2 post-treatment. However, few ssDNA-positive cells were detected in the cerebral cortex on day 1 post TMT treatment.

**TMT treatment produced neuronal death in primary cultures of cortical neurons**

Primary cultures of cortical neurons from mouse embryos were prepared and cultured for 9–12 DIV. Under the culture conditions used, MAP-2–positive cells increased in a culture time–dependent manner, representing more than 80% of the cultured cells on 3–12 DIV [positive cells (% of total cells): 3 DIV, 86.4; 6 DIV, 93.9; 9 DIV, 92.1]. The majority of the resting cells were GFAP-positive cells. A continuous exposure of the cultures to TMT at a concentration of 5 μM produced neuronal damage in an incubation time–dependent manner with a significant effect at an exposure time of 12 h and longer (Fig. 2). Coincident with a retraction of dendrites, as observed by immunostaining for MAP2 in morphological assessments (Fig. 2a), a significant decrease in the β-tubulin III level and a slight increase in the GFAP level were observed when the cell were exposed to TMT for more than 12 h (Fig. 2b). Hoechst 33342 staining revealed that in cultures without TMT, damaged cells with nuclear condensation were 25% and 33% of the total cells at 9 and 12 DIV, respectively. However, the exposure of cultures at 9 DIV to TMT produced a dramatic increase in damaged cells with nuclear condensation in an incubation time–dependent manner at least after exposure for more than 12 h (Fig. 2c). A 48-h exposure to TMT produced damage in more than 90% of the total cells, indicating...
that TMT had the ability to facilitate neuronal damage in the cortical neurons.

*TMT toxicity was independent of N-methyl-d-aspartate (NMDA) excitotoxicity in primary cultures of cortical neurons*

The release of glutamate from neurons is proposed as a possible mechanism underlying TMT toxicity in the central nervous system (27). Indeed, opening of NMDA receptors, which allows Ca$^{2+}$ influx into the cells, causes neuronal death in cortical neurons (28). To evaluate if NMDA excitotoxicity was involved in TMT toxicity in the cultures of cortical neurons, we examined the effects of the NMDA-channel blocker MK-801 on TMT-induced neuronal damage. MK-801 at the concentrations of 10 and 100 μM failed to prevent neuronal damage induced by TMT (Fig. 3). MK-801 alone did not affect cell survival in the absence of TMT. Thus, under these culture conditions, TMT caused neuronal damage independent of NMDA excitotoxicity.
TMT treatment elevated levels of SEK1 and p-SEK1 prior to neuronal death in primary cultures of cortical neurons

SEK1 is a kinase belonging to the mitogen-activated protein kinase kinase family and a specific activator of its downstream kinase, JNK (29). As a first approach to examine the possible activation of JNK cascades prior to neuronal damage induced by TMT, we determined the levels of SEK1 and p-SEK1 after various periods of TMT treatment. The SEK1 level remained unchanged at least after a 6-h exposure to TMT. However, a significant decrease in it was seen after exposure times of 12 and 24 h, at which time neuronal damage was observed (Fig. 4a, lower). Interestingly, the level of p-SEK was significantly elevated after 3 – 6-h exposure to TMT, which elevation was followed by a progressive decrease at 12 – 24 h (Fig. 4a, upper). The relative phosphorylation of SEK1 (p-SEK1/SEK1), which was calculated by dividing units of p-SEK1 level by SEK1 level at each exposure time, rose significantly after the exposure times of 3 – 16 h, which was prior to neuronal death induced by TMT (Fig. 4b).

TMT treatment transiently elevated the levels of JNKs and p-JNKs prior to neuronal death in primary cultures of cortical neurons

JNKs are encoded by 3 genes, jnk1, jnk2, and jnk3, which are spliced alternatively at their 3’-region to create 2 types of proteins. These are termed p46 (JNK$^{p46}$) and p54 (JNK$^{p54}$) depending on their molecular size (30). To investigate whether JNKs were activated in cortical neurons exposed to TMT, we determined the level of JNKs and its active form (p-JNKs) in cortical cultures exposed to TMT. The levels of JNK$^{p54}$ and p-JNK$^{p54}$ in the cultured cells were unaffected during a 12-h exposure, but significantly decreased after exposure

![Fig. 4. Effects of TMT treatment on the level of SEK1 and p-SEK1 in the cortical cultures. Cortical cultures at 9 DIV were exposed to 5 μM TMT for the various periods indicated. a: After each exposure period, the cells were lysed and then subjected to immunoblotting for SEK1 and p-SEK1 to determine the expression and phosphorylation levels of SEK1. b: Relative phosphorylation of p-SEK1 phosphorylation level against SEK1 expression level was calculated at each exposure time. Values are the mean ± S.E.M. from 4 independent experiments. Panels located immediately below the left graphs are typical data from immunoblot analysis. *P<0.05, **P<0.01, significantly different from each control value obtained at 24 h for the cells incubated without TMT (exposure time = C).]

![Fig. 5. Effects of TMT treatment on the level of JNKs and p-JNKs in the cortical cultures. Cortical cultures at 9 DIV were exposed to 5 μM TMT for the various periods indicated. a and c: After each exposure period, the cells were lysed and then subjected to immunoblotting for JNKs and p-JNKs to determine the expression and phosphorylation levels of JNKs (a, p54; c, p46). b and d: Relative phosphorylation of p-JNK phosphorylation level against JNK expression level was calculated at each exposure time (b, p54; d, p46). Values are the mean ± S.E.M. from 4 independent experiments. Panels located immediately below the left graphs are typical data from immunoblot analysis. *P<0.05, **P<0.01, significantly different from each control value obtained at 24 h for the cells incubated without TMT (exposure time = C).]
for 16 – 24 h (Fig. 5a). The relative phosphorylation of JNK<sup>p46</sup> (p-JNK<sup>p46</sup>/JNK<sup>p46</sup>) was not altered at least during a 16-h exposure to TMT (Fig. 5b). TMT transiently elevated the level of p-JNK<sup>p46</sup> after 3- or 6-h exposure. Thereafter the level was decreased after the exposure times of 16 and 24 h (Fig. 5c, upper). However, no significant change was observed in the level of JNK<sup>p46</sup> during the 24-h exposure to TMT (Fig. 5c, lower). The relative phosphorylation of JNK<sup>p46</sup> (p-JNK<sup>p46</sup>/JNK<sup>p46</sup>) was transiently elevated by TMT exposure and then progressively decreased by 24 h of exposure (Fig. 5d).

**TMT treatment dramatically elevated the levels of c-Jun and p-c-Jun in primary cultures of cortical neurons**

c-Jun is an immediately early gene product produced in response to extra- and/or intracellular signals and is involved in multiple cellular functions. These include survival, differentiation, regeneration, and neuronal apoptosis (13). We thus determined the levels of c-Jun and p-c-Jun in the cultures after exposure to TMT for different periods of time. TMT produced a marked elevation of the c-Jun level throughout the exposure period of at least 6 – 24 h (Fig. 6a, lower). Although p-c-Jun was not detectable in untreated cells, TMT exposure led to a dramatic time-dependent (from 3 – 24 h) elevation of its level (Fig. 6a, upper). The relative phosphorylation of c-Jun (p-c-Jun/c-Jun) was markedly elevated by TMT with a peak at 12 h (Fig. 6b).

**Effects of JNK inhibitor on TMT-induced neuronal death in primary cultures of cortical neurons**

To evaluate involvement of JNKs in TMT-induced death of cortical neurons, we assessed the effect of SP600125, an inhibitor of JNKs, on TMT toxicity toward these cells. As shown in Fig. 1, a 24-h exposure of the cultures to 5 µM TMT produced nuclear condensation in more than 90% of the total cells. SP600125 at the concentration of 10 µM was effective in completely inhibiting phosphorylation of c-Jun proteins (Fig. 7a), but only partially prevented the neuronal damage induced by TMT, without affecting spontaneous damage (Fig. 7b). Cells treated with increased concentration (50 µM) of the inhibitor showed neuronal damage similar to that observed at 10 µM (data not shown). In addition to Hoechst 33342 staining, we determined LDH accumulation in the culture medium to assess cell death in the cultures. The accumulation of LDH in the medium was markedly increased by an exposure to TMT for 48 h, at which time complete neuronal loss was observed in the cultures. The LDH accumulation in the medium was significantly but only partly abolished by SP600125 (Fig. 7c). However, SP600125 was incapable of decreasing LDH that had spontaneously accumulated in the medium.

**Discussion**

Previous reports demonstrated that TMT produced neuronal death in the dentate gyrus, but not in the CA subfield, of the hippocampus, olfactory bulb, and anterior olfactory nucleus in adult mice (4 – 9). In the present study, we first demonstrated that TMT was effective in damaging cells in the cerebral frontal cortex in addition to the other portions of the brain mentioned above. To elucidate the mechanisms underlying neuronal death induced by TMT, we used the primary cultures of cortical neurons derived from mouse embryos. The present study showed that a JNK cascade mediated by JNK<sup>p46</sup> was activated prior to neuronal death induced by TMT. These findings strongly support the previous data that a systemic injection of TMT activated the JNK pathway prior to neuronal death in the hippocampal dentate gyrus in vivo (24). In addition, our current study provides data showing that TMT-induced neuronal death was prevented by SP600125. However, this inhibitor of JNKs was effective in only partially preventing both nuclear condensation and LDH accumulation in the medium induced by TMT. Thus, it is likely that the JNK pathway is at least one of the involved mechanisms, but...
not the only one underlying neuronal death induced by TMT. To our knowledge, our current study has provided the first evidence for the involvement of the JNK pathway in TMT-induced damage of cortical neurons.

A principal question arises as to what activated the JNK cascade in neurons exposed to TMT. The JNK pathway is known to be activated in response to glutamate (17). Indeed, previous studies had proposed the possibility that TMT-induced neuronal cell death would be caused by excitotoxicity. TMT enhances endogenous glutamate release from hippocampal slices (27) and also reduces glutamate uptake (31). Moreover, another organotin, tributyltin, elicited the opening of glutamate-receptor channels through the release of glutamate in primary neuronal cultures of rat cerebral cortex (32). In the present study, however, MK-801 failed to block TMT-induced neuronal damage, although the cultured neurons used in the present study had NMDA-receptor subunits including NR1, NR2A, NR2B, NR2C, and NR2D and indeed were vulnerable to a transient exposure to glutamate or NMDA (28). Thus, it is unlikely that excitotoxicity contributed to the TMT-induced neuronal death in the cultures of cortical neurons in the present study. This proposition may be supported by a previous report indicating that tributyltin and triphenyltin, but not TMT, produce an elevation of the intracellular Ca\(^{2+}\) level through enhanced Ca\(^{2+}\) influx and release from intracellular stores in PC12 cells (33). Thus, we propose that the TMT-induced activation of the JNK cascades was not due to glutamate excito-

toxicity in the cortical neurons.

Although the initial molecular action of TMT in neurons encountering this organotin is fundamentally unknown, findings of TMT toxicity in the central nervous system have been accumulating. Compared to immortalized cell lines, primary neuronal cultures are very sensitive to organotins and easily undergo apoptotic cell death (34). In addition to the finding that primary cultures of astrocytes are much more resistant to TMT than neurons, neuronal damage induced by TMT is known to be attenuated when neurons are co-cultured with astrocytes (35). Thus, these findings raise the proposition that astrocytes have a protective effect against TMT-induced neurotoxicity. Nevertheless, TMT has been shown to cause the release of tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) from glial cells including astrocytes (36) and microglia (37, 38). In triple-cultures consisting of neurons, astrocytes, and microglia, TMT produces neuronal death related to TNF-\(\alpha\), which is released from microglia. The TNF-\(\alpha\)-induced neuronal death is blocked by S100\(\beta\), which is derived from astrocytes (38). In the present study, however, TMT-induced neuronal death was not due to the release of factors derived from glial cells because more than 90% of the living cells were neuronal cells. Indeed, we determined no significant change in the level of TNF-\(\alpha\) mRNA during TMT exposure under the current experimental conditions (data not shown). As mentioned above, direct effects of TMT on neurons are fundamentally still unknown. Our findings do not explain the exact mechanism; however,
we propose that TMT directly activated the JNK cascade that contributed to the subsequent cell death of these relatively pure neurons.

JNKs conserve 2 phosphorylation sites at threonine 183 and tyrosine 185, in the amino acid numbering system based on the sequence of JNK1. Of these 2 residues, SEK1 or MKK7 preferentially phosphorylates the tyrosine or threonine residue, respectively. The evidence that the single gene, sek1 or mkk7, deficient cells failed to activate JNK indicates that SEK1 acts on JNK phosphorylation in concert with MKK7 (29). JNK1 phosphorylated at both tyrosine and threonine is 5–10-fold more active than JNK1 phosphorylated at the threonine site alone, whereas JNK1 phosphorylated at the tyrosine site alone is inactive (39). In the present paper, the merged phosphorylation of SEK1 and JNKp46 suggests that SEK1 is an upstream trigger of JNKs cascades activated by TMT.

There are 3 closely related JNK genes (JNK1, JNK2, and JNK3) that give rise to different splice variants resulting in 10 potential isoforms in the adult human brain (30). Electrophoretic analysis demonstrates the presence of 2 classes of JNKs, which have apparent molecular weights of 46 and 54 kDa. Alternative processing of RNA transcripts results in the deletion or retention of 5 nucleotides in the coding region for the JNK COOH-terminus of JNK1, JNK2, and JNK3 genes. Thus, JNKs with molecular weights of 46 and 54 kDa are mixtures of products from splicing variants of JNK1, JNK2, and JNK3 genes. Our present data that TMT was effective in enhancing the phosphorylation of JNKp46, but not JNKp54, lead us to speculate that TMT stimulated the phosphorylation of JNKs encoded by alternative variants with deletion or retention in the JNK COOH-terminus of JNK1, JNK2, and JNK3. The findings that TMT decreased the level of JNKp54 protein, but not that of the JNKp46 protein or c-Jun protein, at relatively late time windows allow us to propose the idea that TMT has the ability to down-regulate the expression of splicing variants encoding JNKp54 protein. However, further studies are needed to elucidate which isoforms of the 10 potential isoforms would be regulated by TMT.

In most situations, extracellular signals as a first messenger activate intracellular second messenger signals at membrane receptors. Second messengers such as cyclic nucleotides and ions are able to trigger signaling cascades in the cytoplasm; and these signals are propagated into the nucleus to express the mRNAs of particular genes, often referred to as immediate early genes (e.g., c-fos, c-jun, c-myc, and egr-1), as third messengers (40). These third messengers encode transcription factors that induce the expression of various target genes. The target genes of c-Jun are involved in cellular proliferation, differentiation, growth for malignancy, and cell death by apoptosis (13, 40). Although TMT increased c-Jun expression and phosphorylation of c-Jun with apoptotic nuclear condensations in the cortical neurons in the present study, the cells could have been dying via both apoptotic and necrotic pathways (41). Further experiments are required to determine which genes are expressed for cell death, such as those for caspases and/or calpains.

The mechanism underlying TMT-induced SEK1 and/or JNK activation is still unclear. One attractive mechanism is that reactive oxygen species are involved in the activation. Indeed, TMT has been shown to induce their production (35). This is further supported by the findings that reactive oxygen species induce the activation of JNKs in a dopaminergic neuronal cell line (42), renal proximal tubule epithelial cells (43), human fibroblasts (44), and murine embryonic fibroblasts (45). These processes could be operative in mechanisms underlying particular neurological disorders with depleted endogenous glutathione as seen in Parkinson’s disease (46) and brain ischemia (47). The neuronal death caused by TMT appears to be useful as an in vivo model for neurochemical, physiological, anatomical, and pharmacological studies on a variety of neurodegenerative disorders associated with neuronal damage, especially those involving the dentate gyrus (20). Evaluation of the underlying mechanisms could give us hints crucial to the discovery and development of therapeutics for various neurodegenerative disorders in human beings.

All the above mentioned findings suggest that a direct exposure to TMT results in neuronal death by the activation of JNK cascades, which involve JNKp46, but not JNKp54. In cortical neurons, it is likely that neuronal death induced by TMT is caused by yet unidentified factors, but at least it is currently indicated that it is not caused by well-known ones such as excitatory amino acids or TNF-α. Our approaches using the TMT toxicity model are important for elucidating the sensitivity of neurons to and/or tolerability toward neurotoxins and hopefully will contribute to defining the mechanisms underlying various neuronal degenerative diseases.

Acknowledgments

This work was supported by Grants-in-Aid for scientific research (to K.O. and N.K.) from the Ministry of Education, Science, Sports, and Culture, Japan.

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